

**Center for Veterinary Biologics  
and  
National Veterinary Services Laboratories  
Testing Protocol**

**Supplemental Assay Method for Titration of Canine  
Parainfluenza Virus in Vero Cell Culture**

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Supplemental Assay Method for Titration of Canine Parainfluenza Virus in Vero Cell Culture

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## 1. Introduction

### 1.1 Background

This Supplemental Assay Method (SAM) describes an *in vitro* test method for assaying modified-live canine parainfluenza (CPI) virus vaccines for viral content. Presence or absence of CPI virus is determined by hemadsorption (HAd) of guinea pig red blood cells (GPRBC).

### 1.2 Keywords

Canine parainfluenza; CPI; HAdID<sub>50</sub>; potency test; titration; GPRBC; *in vitro*

## 2. Materials

### 2.1 Equipment/instrumentation

2.1.1 Incubator,<sup>1</sup> 36° ± 2°C, high humidity, 5% ± 1% CO<sub>2</sub> meeting the requirements of the current version of GDOCSOP004

2.1.2 Water bath,<sup>2</sup> 36° ± 2°C

2.1.3 Centrifuge<sup>3</sup> and rotor<sup>4</sup>

2.1.4 Microscope,<sup>5</sup> inverted light

2.1.5 Vortex mixer<sup>6</sup>

2.1.6 Syringe,<sup>7</sup> self-refilling repetitive, 2 ml

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<sup>1</sup> Model 3336, Forma Scientific, Inc., P.O. Box 649, Marietta, OH 45750 or equivalent

<sup>2</sup> Cat. No. 66648, Precision Scientific, 3737 West Cortland St., Chicago, IL 60647 or equivalent

<sup>3</sup> Model J6-B, Beckman Coulter, Inc., P.O. Box 3100, Fullerton, CA 92834-3100 or equivalent

<sup>4</sup> JS-4.0, Beckman Coulter, Inc. or equivalent

<sup>5</sup> Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent

<sup>6</sup> Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent

<sup>7</sup> Wheaton®, Cat. No. 13-689-50C, Fisher Scientific Co., 319 W. Ontario, Chicago, IL 60610 or equivalent

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2.1.7 Pipettor<sup>8</sup> with tips<sup>9</sup> and/or motorized microliter pipette<sup>10</sup> and tips<sup>11</sup>

2.1.8 Pipette-aid<sup>12</sup>

## 2.2 Reagents/supplies

2.2.1 CPI Virus Reference, D008 strain<sup>13</sup>

2.2.2 Monospecific antisera,<sup>14</sup> free of CPI antibody, which neutralize the non-CPI virus fractions present in multifraction vaccines (e.g., canine distemper virus [CDV] and canine adenovirus [CAV])

2.2.3 African green monkey kidney (Vero) cell line,<sup>15</sup> free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

2.2.4 Minimum essential medium (MEM)

2.2.4.1 9.61 g MEM<sup>16</sup> with Earle's salts

2.2.4.2 2.2 g sodium bicarbonate (NaHCO<sub>3</sub>)<sup>17</sup>

2.2.4.3 Dissolve reagents in **Sections 2.2.4.1-2.2.4.2** with 900 ml deionized water (DW).

2.2.4.4 Add 5.0 g lactalbumin hydrolysate or edamin<sup>18</sup> to 10 ml DW, and heat to 60° ± 2°C until dissolved. Add to the solution in **Section 2.2.4.3** with constant mixing.

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<sup>8</sup> Cat. No. P-200, Rainin Instrument Co., P.O. Box 4026, Mack Rd., Woburn, MA 01801-4628 or equivalent

<sup>9</sup> Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY 11571 or equivalent

<sup>10</sup> Cat. No. E2-1000, Rainin Instrument Co. or equivalent

<sup>11</sup> Cat. No. RT-200, Analytic Lab Accessories or equivalent

<sup>12</sup> Cat. No. 183, Drummond Scientific Co., 500 Pkwy., Broomall, PA 19008 or equivalent

<sup>13</sup> Available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent

<sup>14</sup> Reference quantities are available upon request from the CVB-L or equivalent

<sup>15</sup> ATCC CCL 81, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852 or equivalent

<sup>16</sup> Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgeman Ct., Gaithersburg, MD 20884 or equivalent

<sup>17</sup> Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

<sup>18</sup> Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

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2.2.4.5 Q.S. to 1000 ml with DW and adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).<sup>19</sup>

2.2.4.6 Sterilize through a 0.22-µm filter.<sup>20</sup>

2.2.4.7 Aseptically add:

1. 25 units/ml penicillin<sup>21</sup>
2. 50 µg/ml gentamicin sulfate<sup>22</sup>
3. 100 µg/ml streptomycin<sup>23</sup>

2.2.4.8 Store at 4° ± 2°C.

2.2.5 Growth Medium

2.2.5.1 940 ml MEM

2.2.5.2 Aseptically add:

1. 50 ml gamma-irradiated fetal bovine serum (FBS)
2. 10 ml L-glutamine<sup>24</sup>

2.2.5.3 Store at 4° ± 2°C.

2.2.6 Alsevers Solution

2.2.6.1 8.0 g sodium citrate (C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>•2H<sub>2</sub>O)<sup>25</sup>

2.2.6.2 0.55 g citric acid (C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>•H<sub>2</sub>O)<sup>26</sup>

2.2.6.3 4.2 g sodium chloride (NaCl)<sup>27</sup>

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<sup>19</sup> Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

<sup>20</sup> Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

<sup>21</sup> Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent

<sup>22</sup> Cat. No. 0061-0464-04, Schering Laboratories or equivalent

<sup>23</sup> Cat. No. S-9137, Sigma Chemical Co. or equivalent

<sup>24</sup> 200 mM (100X) liquid, Cat. No. G-7513, Sigma Chemical Co. or equivalent

<sup>25</sup> Cat. No. S-4641, Sigma Chemical Co. or equivalent

<sup>26</sup> Cat. No. C-7129, Sigma Chemical Co. or equivalent

<sup>27</sup> Cat. No. 3624-01, J.T. Baker, Inc. or equivalent

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2.2.6.4 20.5 g glucose ( $C_6H_{12}O_6$ )<sup>28</sup>

2.2.6.5 Q.S. to 1000 ml with DW and dissolve.

2.2.6.6 Sterilize through a 0.22- $\mu$ m filter.

2.2.6.7 Store at  $4^\circ \pm 2^\circ C$ .

2.2.7 Dulbecco's phosphate buffered saline free of calcium chloride and magnesium chloride ( $Ca^{++}$ ,  $Mg^{++}$  Free DPBS)

2.2.7.1 8.0 g NaCl

2.2.7.2 0.2 g potassium chloride (KCl)<sup>29</sup>

2.2.7.3 0.2 g potassium phosphate, monobasic, anhydrous ( $KH_2PO_4$ )<sup>30</sup>

2.2.7.4 Dissolve reagents in **Section 2.2.7.1** through **Section 2.2.7.3** with 900 ml DW.

2.2.7.5 Add 1.03 g sodium phosphate, dibasic, anhydrous ( $Na_2HPO_4$ )<sup>31</sup> to 10 ml DW, and heat to  $60^\circ \pm 2^\circ C$  until dissolved. Add to **Section 2.2.7.4** with constant mixing.

2.2.7.6 Q.S. to 1000 ml with DW; adjust pH to 7.0-7.3 with 2N HCl.

2.2.7.7 Sterilize through a 0.22- $\mu$ m filter.

2.2.7.8 Store at  $4^\circ \pm 2^\circ C$ .

2.2.8 Dulbecco's phosphate buffered saline (DPBS)

2.2.8.1 8.0 g NaCl

2.2.8.2 0.2 g KCl

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<sup>28</sup> Cat. No. G-8270, Sigma Chemical Co. or equivalent

<sup>29</sup> Cat. No. P217-500, Fisher Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219-4785 or equivalent

<sup>30</sup> Cat. No. 3246-01, J.T. Baker, Inc. or equivalent

<sup>31</sup> Cat. No. 3828-01, J.T. Baker, Inc. or equivalent

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**2.2.8.3** 0.2 g  $\text{KH}_2\text{PO}_4$

**2.2.8.4** 0.1 g magnesium chloride, hexahydrate  
( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ )<sup>32</sup>

**2.2.8.5** Dissolve reagents in **Section 2.2.8.1**  
through **Section 2.2.8.4** with 900 ml DW.

**2.2.8.6** Add 1.03 g  $\text{Na}_2\text{HPO}_4$  to 10 ml DW, and heat  
to  $60^\circ \pm 2^\circ\text{C}$  until dissolved. Add to **Section**  
**2.2.8.5** with constant mixing.

**2.2.8.7** Dissolve 0.1 g calcium chloride,  
anhydrous ( $\text{CaCl}_2$ )<sup>33</sup> with 10 ml DW, and add slowly  
to **Section 2.2.8.5** to avoid precipitation.

**2.2.8.8** Q.S. to 1000 ml with DW; adjust pH to  
7.0-7.3 with 2N HCl.

**2.2.8.9** Sterilize through a 0.22- $\mu\text{m}$  filter.

**2.2.8.10** Store at  $4^\circ \pm 2^\circ\text{C}$ .

**2.2.9** GPRBC in an equal volume of Alsevers Solution

**2.2.10** Cell culture plates,<sup>34</sup> 24 well

**2.2.11** Polystyrene tubes,<sup>35</sup> 12 x 75 mm

**2.2.12** Pipettes, 10 ml<sup>36</sup> and 25 ml<sup>37</sup>

**2.2.13** Conical tubes,<sup>38</sup> 50 ml

**2.2.14** Syringe,<sup>39</sup> 1 ml tuberculin, and needle,<sup>40</sup>  
18 ga x 1½ in

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<sup>32</sup> Cat. No. M33-500, Fisher Scientific Co. or equivalent

<sup>33</sup> Cat. No. 4225-05, J.T. Baker, Inc. or equivalent

<sup>34</sup> Cat. No. 3524, Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent

<sup>35</sup> Falcon® 2058 and 7530 respectively, Becton Dickinson Labware, 2 Oak Park, Bedford, MA 01730  
or equivalent

<sup>36</sup> Cat. No. 72105-10110, Owens Illinois, Kimble Division, Crystal Ave., Vineland, NJ 08360 or  
equivalent

<sup>37</sup> Cat. No. 4251, Costar Corp. or equivalent

<sup>38</sup> Cat. No. 62.547.205, Sarstedt Inc., P.O. Box 468, Newton, NC 28658-0468 or equivalent

<sup>39</sup> Cat. No. 309602, Becton Dickinson & Co., 1 Becton Dr., Franklin Lakes, NJ 07417-1884 or  
equivalent

<sup>40</sup> Cat. No. 305196, Becton Dickinson & Co. or equivalent

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### **3. Preparation for the test**

#### **3.1 Personnel qualifications/training**

Personnel shall have experience in the preparation and maintenance of cell culture as well as in the propagation and maintenance of animal viruses and the quantitation of virus infectivity by HAd.

#### **3.2 Preparation of equipment/instrumentation**

On the day of test initiation, set a water bath at  $36^{\circ} \pm 2^{\circ}\text{C}$ .

#### **3.3 Preparation of reagents/control procedures**

##### **3.3.1 Preparation of Vero cell culture plates (Test Plates)**

Cells are prepared from healthy, confluent Vero cells that are maintained by passing every 3 to 4 days. One day prior to test initiation, using a self-refilling repetitive syringe, add 1.0 ml/well of  $10^{4.7}$  to  $10^{5.2}$  cells/ml cells suspended in Growth Medium into all wells of a 24-well cell culture plate. Prepare 1 Vero plate for the controls and 1 for each Test Serial. These become the test plates. Incubate at  $36^{\circ} \pm 2^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator for  $24 \pm 12$  hr.

##### **3.3.2 Preparation of CPI Virus Reference Control**

**3.3.2.1** On the day of test initiation, rapidly thaw a vial of CPI Virus Reference in a water bath.

**3.3.2.2** Using a self-refilling repetitive syringe, dispense 1.8 ml MEM into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB-L Reference and Reagent sheet; appropriately label (e.g., 9 tubes, labeled  $10^{-1}$  through  $10^{-9}$ , respectively).

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**3.3.2.3** With a 200- $\mu$ l pipettor, transfer 200  $\mu$ l of the CPI Virus Reference to the first tube labeled  $10^{-1}$ ; mix by vortexing.

**3.3.2.4** Using a new pipette tip, transfer 200  $\mu$ l from the  $10^{-1}$  labeled tube (**Section 3.3.2.3**) to the  $10^{-2}$  tube; mix by vortexing.

**3.3.2.5** Repeat **Section 3.3.2.4** for each of the subsequent dilutions, transferring 200  $\mu$ l of the previous dilution to the next dilution tube until the tenfold dilution series is completed.

**3.3.3** Prepare a dilution of each neutralizing non-CPI antiserum in DPBS according to the supplied CVB-L Reference and Reagent sheet or as determined for that specific antiserum.

**3.3.4** Preparation of GPRBC

**3.3.4.1** Within 7 days of conducting the HAd protocol, transfer the GPRBC into a 50-ml conical tube.

**3.3.4.2** Q.S. to 50 ml with Alsevers Solution, and mix by inverting several times.

**3.3.4.3** Centrifuge for  $15 \pm 5$  min at  $400 \times g$  (1500 rpm in a Beckman J6B centrifuge with JS-4.0 rotor).

**3.3.4.4** Remove supernatant and buffy coat by pipetting.

**3.3.4.5** Repeat steps in **Sections 3.3.4.2-3.3.4.4** for a total of 3 washes.

**3.3.4.6** Add an equal volume of Alsevers Solution. Store at  $4^{\circ} \pm 2^{\circ}\text{C}$ ; use within 1 wk of collection of the GPRBC.

**3.3.5** 0.5% GPRBC Suspension for the HAd assay

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**3.3.5.1** On the day of conducting the HAD protocol, pipette 500  $\mu$ l of washed, packed GPRBC into 100 ml of  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ -Free DPBS, rinsing the pipette thoroughly to remove the GPRBC.

**3.3.5.2** Mix by inverting, store at  $4^{\circ} \pm 2^{\circ}\text{C}$ , and use within 1 wk of collection of the GPRBC.

**3.4 Preparation of the sample**

**3.4.1** Conduct the initial test of a Test Serial with a single vial (a single sample from 1 vial). On the day of inoculation, using a sterile 1.0-ml syringe and an 18-ga x  $1\frac{1}{2}$ -in needle, rehydrate a vial of the Test Serial with the provided diluent by transferring 1.0 ml for 1-ml-dose vaccines, 0.5 ml for 0.5-ml-dose vaccines, etc., into the vial containing the lyophilized Test Serial; mix by vortexing. Incubate for  $15 \pm 5$  min at room temperature (RT) ( $23^{\circ} \pm 2^{\circ}\text{C}$ ).

**3.4.2** For multifraction CPI vaccines, neutralize the non-CPI viral fractions with antiserum specific to each non-CPI virus fraction. It is not necessary to neutralize canine parvovirus (CPV) since CPV is not expected to replicate in Vero cells.

**3.4.2.1** Dispense 200  $\mu$ l of each of the required neutralizing antiserum into a 12 x 75-mm polystyrene tube labeled  $10^{-1}$ , and q.s. with MEM to 1.8 ml. For example, to neutralize 2 non-CPI viral fractions in a 3-fraction CDV/CPI/CAV vaccine, dispense 200  $\mu$ l of each of the diluted CDV and CAV antisera into the tube labeled  $10^{-1}$ ; add 1.4 ml of MEM to obtain a final volume of 1.8 ml.

**3.4.2.2** Pipette 200  $\mu$ l of the reconstituted Test Serial to the  $10^{-1}$  labeled tube containing the neutralizing antisera; mix by vortexing.

**3.4.2.3** Incubate at RT for  $60 \pm 10$  min.

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**3.4.3** For Test Serials containing only the CPI viral fraction, prepare the  $10^{-1}$  dilution by mixing 200  $\mu$ l of the reconstituted Test Serial with 1.8 ml of MEM in a labeled 12 x 75-mm polystyrene tube.

**3.4.4** Serial tenfold dilutions

**3.4.4.1** Using a 2-ml self-refilling repetitive syringe, dispense 1.8 ml MEM into each of 5, 12 x 75-mm polystyrene tubes labeled  $10^{-2}$  through  $10^{-6}$  (or more if the expected CPI endpoint of the Test Serial is higher than  $10^{-6}$ ).

**3.4.4.2** Using a new pipette tip, transfer 200  $\mu$ l from the tube labeled  $10^{-1}$  to the next dilution tube; mix by vortexing.

**3.4.4.3** Repeat **Section 3.4.4.2** to the remaining tubes, transferring 200  $\mu$ l from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

**4. Performance of the test**

**4.1** On the day of test inoculation, label the Test Plates and inoculate each of 5 wells/dilution with 200  $\mu$ l of dilutions  $10^{-6}$  through  $10^{-3}$  of each Test Serial. In a similar manner, identify and inoculate 5 wells/dilution of the CPI Virus Reference Control (with dilutions  $10^{-9}$  through  $10^{-6}$  for the example in **Section 3.3.2**). Change tips between each unique sample (i.e., each Test Serial and the CPI Virus Reference Control); tip changes are not necessary between dilutions in a series if pipetting from the most dilute to the most concentrated within that series (e.g.,  $10^{-9}$  through  $10^{-6}$ ).

**4.2** Five uninoculated wells serve as Negative Cell Controls.

**4.3** Incubate the Test Plates in a  $36^{\circ} \pm 2^{\circ}\text{C}$   $\text{CO}_2$  incubator for 8 days  $\pm$  1 day.

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**4.4** After incubation, perform the HAd assay on the cell monolayers with a 0.5% GPRBC suspension.

**4.4.1** Decant the media from the plates into an autoclavable container.

**4.4.2** Submerge each Test Plate in a reservoir of  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ -Free DPBS and decant.

**4.4.3** Mix the 0.5% GPRBC suspension by gentle inversion, and add 1.0 ml/well.

**4.4.4** Incubate at  $4^{\circ} \pm 2^{\circ}\text{C}$  for  $25 \pm 5$  min.

**4.4.5** Repeat **Section 4.4.2** twice.

**4.4.6** Read the wet Test Plate at 100X magnification on an inverted light microscope, and examine cells for HAd.

**4.4.6.1** Wells displaying GPRBC adsorbed onto the cell monolayer are considered to be positive for CPI.

**4.4.6.2** Record the results as the number of HAd-positive wells versus total number of wells examined for each dilution of the Test Serial and the CPI Virus Reference Control.

**4.5** Calculate the CPI endpoints of the Test Serial and the CPI Virus Reference Control using the Spearman-Kärber method as modified by Finney. The titers are expressed as  $\log_{10}$  50% HAd infective doses ( $\text{HAdID}_{50}$ ).

Example:

$10^{-3}$  dilution of Test Serial = 5/5 wells HAd positive  
 $10^{-4}$  dilution of Test Serial = 5/5 wells HAd positive  
 $10^{-5}$  dilution of Test Serial = 1/5 wells HAd positive  
 $10^{-6}$  dilution of Test Serial = 0/5 wells HAd positive

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Spearman-Kärber calculation of total HAd-positive wells  
(11), using 5 wells = 1.7 log

Log<sub>10</sub> of reciprocal dilution (10<sup>-3</sup>) = 3.0 log

Log<sub>10</sub> of reciprocal of dose factor:

$\frac{0.2 \text{ ml inoculum}}{1\text{-ml dose}} = \frac{1}{5} = 0.7 \text{ log}$

Total = 5.4 log

Titer is 10<sup>5.4</sup> HAdID<sub>50</sub>.

## 5. Interpretation of the test results

### 5.1 Validity requirements

**5.1.1** The calculated titer of the CPI Virus Reference Control must fall within plus or minus two standard deviations ( $\pm 2$  SD) of its mean titer, as established from a minimum of 10 previously determined titers.

**5.1.2** The lowest inoculated dilution of the CPI Reference Control must exhibit a 100% positive HAd reaction (5/5), and the highest (most dilute) must exhibit no positive HAd reaction (0/5).

**5.1.3** The Negative Cell Controls shall not exhibit degradation, HAd, or cloudy media indicative of contamination.

**5.2** If the validity requirements are not met, the assay is considered a **NO TEST** and can be retested without prejudice.

**5.3** In a valid test, if the titer of the Test Serial is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production for the product under test, the Test Serial is considered **SATISFACTORY**.

**5.4** In a valid test, if the titer of the Test Serial is less than the titer contained in the APHIS filed Outline of

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Production for the product under test, the Test Serial may be retested in accordance with 9 CFR, Part 113.8.

## 6. Report of test results

Results are reported as HAdID<sub>50</sub> per dose of Test Serial.

## 7. References

7.1 Code of Federal Regulations, Title 9, Part 113.316, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Cottral, GE, (Ed.), 1978. *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, pg. 731.

7.3 Finney, DJ, 1978. *Statistical method in biological assay*. Griffin, London. 3rd edition, pg. 508.

## 8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, to provide additional detail, and to reflect the change in the procedure for neutralizing non-CPI viral fractions from the superseded protocol.